Small Molecule Screen Identifies Non-Catalytic USP3 Chemical Handle

Mandeep K. Mann,[‡,†], *Brian Wilson*[$], *Rachel J. Harding**[‡,†], *Matthieu Schapira**[‡,†]

[‡]Structural Genomics Consortium, University of Toronto, 101 College Street, MaRS South Tower, Suite 700, Toronto, Ontario M5G 1L7, Canada

[†]Department of Pharmacology and Toxicology, University of Toronto, 1 King's College Circle, Toronto, Ontario M5S 1A8, Canada

[$] Drug Discovery Program, Ontario Institute for Cancer Research, Toronto, Ontario M5G 0A3, Canada

*Corresponding Author

ABSTRACT

Zinc-finger ubiquitin binding domains (ZnF-UBDs) are non-catalytic domains mostly found in deubiquitinases (DUBs). They represent an underexplored opportunity for the development of deubiquitinase-targeting chimeras (DUBTACs) to pharmacologically induce the deubiquitination of target proteins. We have previously shown that ZnF-UBDs are ligandable domains. Here, a focused small molecule library screen against a panel of eleven ZnF-UBDs led to the identification of **59**, a ligand selective for USP3 ZnF-UBD that does not inhibit USP3 catalytic

activity. A preliminary structure activity relationship of the chemical series informs future development of molecules targeting the USP3 ZnF-UBD domain. The work reported here can be used to further explore the function of the USP3 ZnF-UBD and to develop bifunctional DUBTACs recruiting USP3 for targeted deubiquitination.

INTRODUCTION

Proximity-induced pharmacology is an expanding field in drug discovery, in which post-translational modifications can be induced through the recruitment of protein-modifying enzymes to target proteins. Mechanistic proof-of-concept was established with proteolysis targeting chimeras (PROTACs) that induce ubiquitination and proteasomal degradation of a target protein via the recruitment of an E3 ubiquitin ligase. Conversely, the ability to pharmacologically rescue proteins from proteasomal degradation can allow the investigation of protein function and may have therapeutic benefits in disease settings where a protein is aberrantly degraded. Bifunctional molecules presenting a chemical handle binding a non-catalytic pocket of a deubiquitinase (DUB), an enzyme that removes ubiquitin groups, may enable targeted protein deubiquitination and subsequent target stabilization. The first reported deubiquitinase-targeting chimera (DUBTAC) recruited OTUB1, a K48 ubiquitin specific DUB[1]. The compound was composed of a covalent ligand binding an allosteric site of OTUB1, linked to a drug that binds a mutant cystic fibrosis transmembrane conductance regulator (CFTR), resulting in stabilized CFTR protein levels in human cystic fibrosis bronchial epithelial cells[1].

Twelve ubiquitin specific proteases (USPs) - a subfamily of DUBs - have a non-catalytic zinc-finger ubiquitin binding domain (ZnF-UBD), a unique fold that primarily binds ubiquitin (Ub) or

ubiquitinated substrates via an unanchored C-terminal Ub RLRGG motif (USP3, USP5, USP13, USP16, USP20, USP22, USP33, USP39, USP44, USP45, USP49, USP51)[2–4]. The ZnF-UBD is also found in HDAC6, a histone deacetylase, and BRAP, a BRCA-1 associated protein and Ub ligase[5–8] (**Figure 1A**). While ZnF-UBDs generally engage Ub with a low micromolar binding affinity, this function is lost in some USPs (USP13, USP20, USP22, USP33, USP39 and USP51) due to key substitutions of ubiquitin-coordinating residues[3,9–11] (**Figure 1B**). We previously demonstrated the ligandability of the ZnF-UBD with low micromolar ligands for the ZnF-UBD of USP5 and HDAC6[12–15] (**Figure 1AC**). The function of ZnF-UBDs and their role in catalytic activity of DUBs is poorly understood. We have shown that ligands targeting the ZnF-UBD of USP5 inhibit its catalytic activity[12,13] and therefore cannot be used as chemical handles for the development of USP5-recruiting DUBTACs, but the same may not be true for other USPs.

Here, we report the discovery and characterization of USP3 ZnF-UBD ligands. Screening of a small, focused chemical library against a panel of ZnF-UBDs led to the discovery of a selective USP3 ligand that does not inhibit the catalytic function of USP3. We also present a preliminary exploration of the structure activity relationship (SAR) of the chemical series. Our work provides a framework for the development of potent USP3 ZnF-UBD ligands as chemical handles for DUBTACs, and as tools to study the function of the USP3 ZnF-UBD in health and disease.

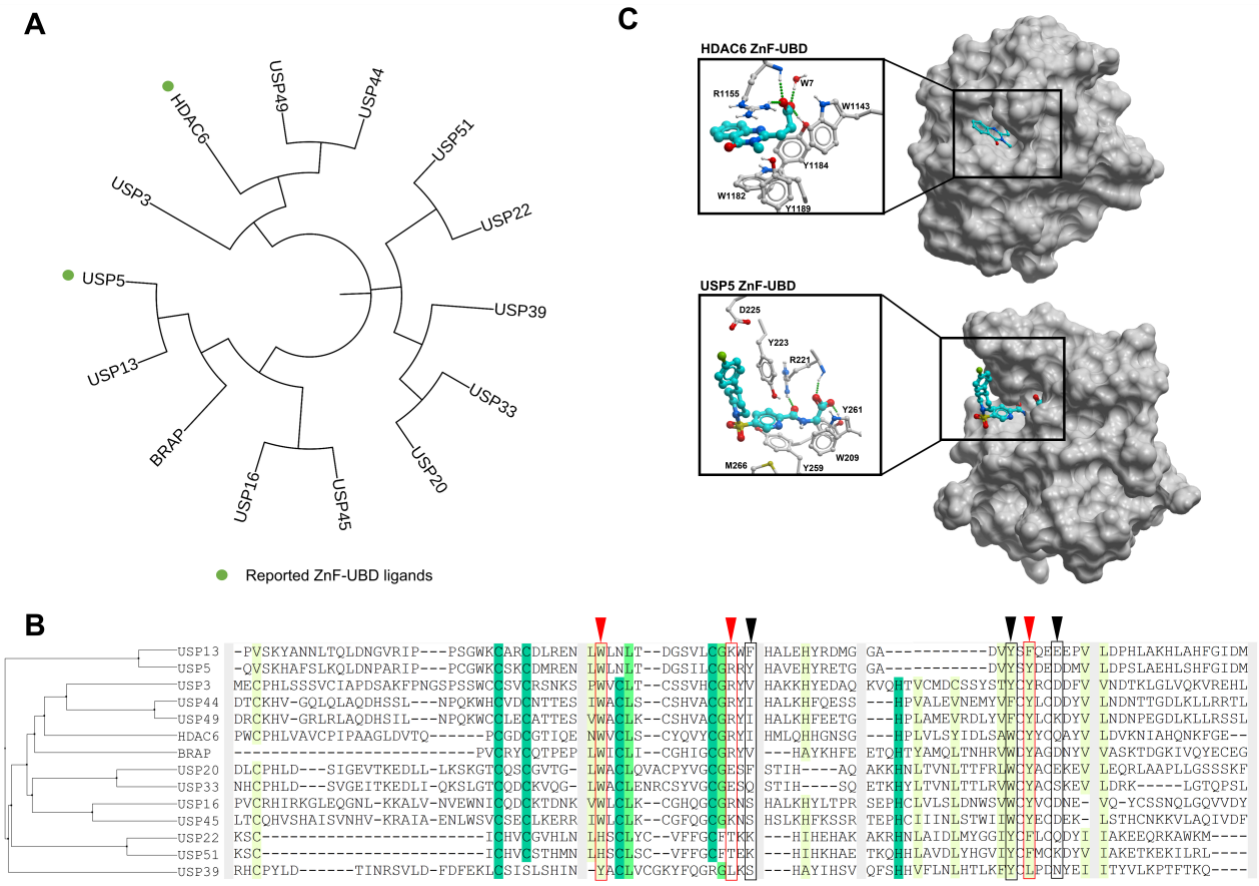
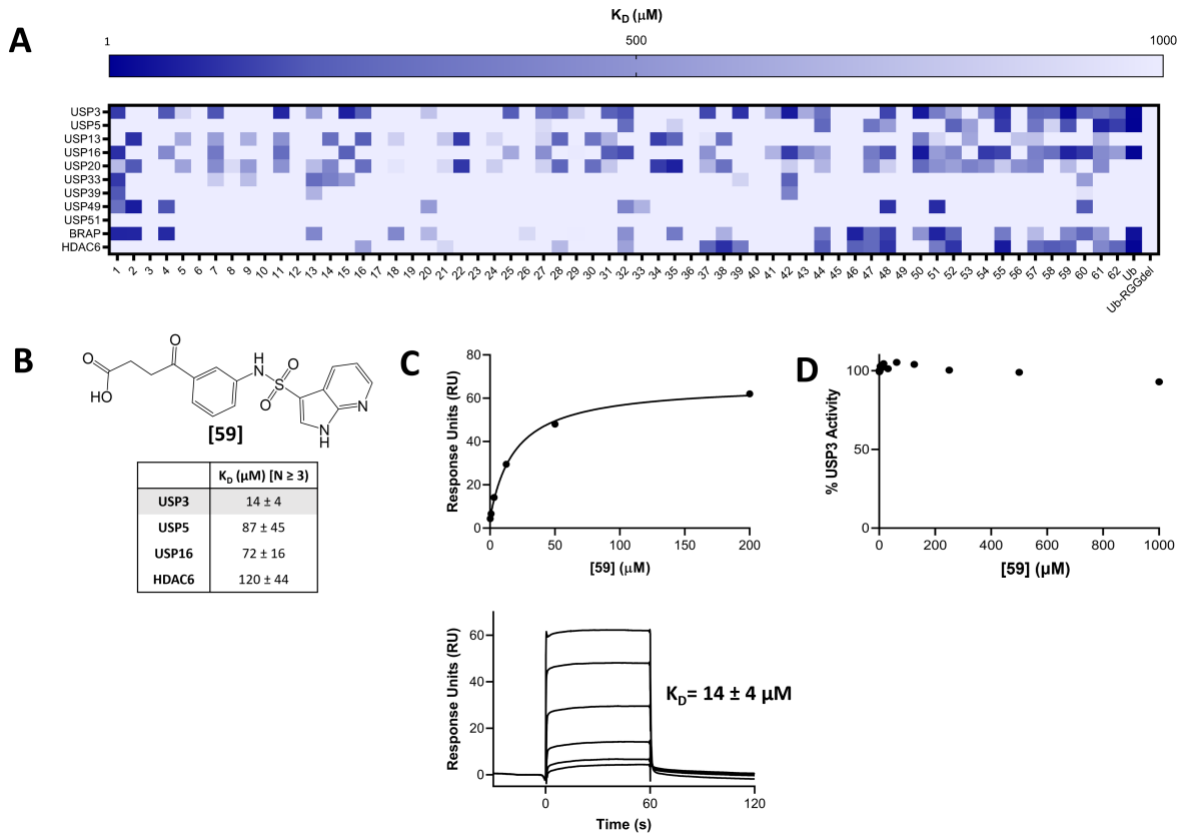


Figure 1. **Human ZnF-UBDs:** A) (Left) Dendrogram of full-length human proteins that contain a ZnF-UBD. The dendrogram was created by first doing a multiple sequence alignment using Clustal Omega[16], followed by tree generation with iTol[17]. Reported ZnF-UBD ligands are highlighted with green circles B) Sequence alignment of human ZnF-UBDs. Conserved residues are indicated with shading. USP5 residues that interact with ubiquitin are indicated with arrowheads and boxes (red: expected to be essential for ubiquitin binding; black: not essential). C) Co-crystal structures of HDAC6 and USP5 ZnF-UBD in complex with low micromolar ligands (PDB: 6CED, 7MS7). Hydrogen bonds are displayed as dashed lines.

RESULTS AND DISCUSSION

Following our observation that an aliphatic chain ending with a carboxylic acid was essential for binding to the ZnF-UBD of HDAC6 and USP5, we compiled a focused library of 670 molecules bearing this moiety from our in-house collection to target the Ub-binding ZnF-UBDs of USP3, USP5, USP13, USP16, USP20, USP22, USP33, USP39, USP49, USP51, BRAP and HDAC6. Compounds were grouped into 62 clusters with ICM (Molsoft) based on their chemical similarity, and the binding of 62 representative molecules to our panel of 11 ZnF-UBDs was measured in a surface plasmon resonance (SPR) assay. Ub and a C-terminal RGG-deleted Ub construct were used as positive and negative controls, respectively (**Figure 2A, Table S1 & S2)**. Compound **59** was identified and confirmed as a promising USP3 hit and was more than 5-times selective for USP3 over USP5, USP16, and HDAC6 ZnF-UBDs (K_D: USP3 = 14 ± 4 μM) (**Figure 2BC, Table 1**). Importantly, **59** did not inhibit USP3 DUB catalytic activity in a fluorogenic ubiquitin rhodamine110 assay **(Figure 2D**).



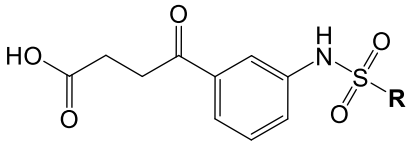
	K_D (μM) [N ≥ 3]
USP3	14 ± 4
USP5	87 ± 45
USP16	72 ± 16
HDAC6	120 ± 44

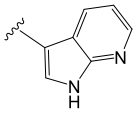
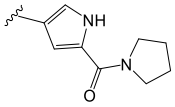
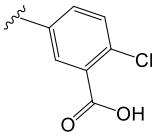
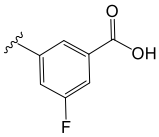
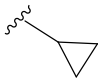
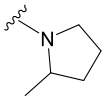
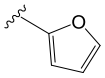
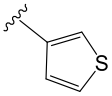
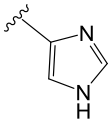
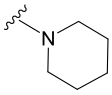
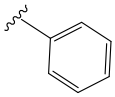
Figure 2. A small molecule screen identifies ZnF-UBD hits. A) A heat map showing binding of 64 ligands to 11 ZnF-UBDs by SPR. A 4-fold 6-point dilution series beginning at 200 μM was used for K_D determination with N=1. B) Summary of binding data for compound **59**, N≥3. C) Representative SPR binding curve and sensorgram for USP3 ZnF-UBD and **59**. A K_D of 14 ± 4 was obtained from the average of seven independent measurements. D) **59** does not inhibit USP3 in a UbRho110 assay (N=2). Fluorescence signal is normalized to control (no compound, DMSO only).

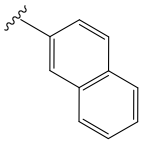
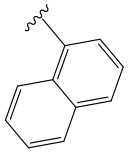
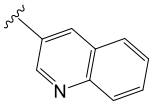
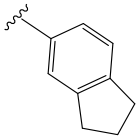
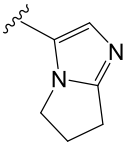
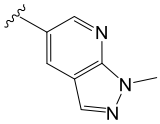
To validate this chemical template, three in-house analogs of **59** were tested for binding to USP3, USP5 and HDAC6 confirming that potency and selectivity varied with substituents attached to the sulfonamide group (**Table 1**, **63-65**). A substructure search of the Enamine REAL database

for analogs of **59** and **63** (K_D = 13 ± 5 µM) resulted in 797 compounds, which were clustered with ICM (Molsoft). Fourteen diverse molecules were ordered and tested for binding to define a preliminary structure activity relationship (SAR) (**Table 1**, **66-79**). While more potent molecules were not identified, this limited SAR exploration showed that hetero-bicyclic rings with a nitrogen in 2-position of the sulfonamide linker are preferred (**59**, **63**, **76, 79**).

Table 1. In-house and Enamine REAL analogs of **59**



Cpd	R	USP3 K_D[µM][a]	USP5 K_D [µM][a]	HDAC6 K_D [µM][a]	Cpd	R	USP3 K_D [µM][a]	USP5 K_D [µM][a]	HDAC6 K_D [µM][a]
59		14 ± 4	87 ± 45	120 ± 44	**63**		13 ± 4	93 ± 33	158 ± 65
64		180 ± 112	>200	>200	**65**		160 ± 76	>200	>200
66		110 ± 25	152 ± 45	194 ± 100	**67**		104 ± 27	>200	>200
68		95 ± 46	195 ± 108	82 ± 41	**69***		141 ± 74	192 ± 75	>200
70*		48 ± 18	78 ± 15	65 ± 10	**71**		69 ± 23	180 ± 73	184 ± 124
72		99 ± 50	183 ± 71	146 ± 72	**73**		76 ±35	113 ± 14	95 ± 12

74		124 ± 49	97 ± 29	105 ± 19	**75**		77 ± 18	53 ± 18	56 ± 12
76		48 ± 30	98 ± 45	87 ± 31	**77**		59 ± 30	159 ± 45	81 ± 14
78*		65 ± 31	>200	102 ± 44	**79**		47 ± 20	113 ± 19	127 ± 27

[a]K_D determination experiments were performed with N ≥ 3 and the values are presented as mean ± SD.

*Some signs of non-specific binding.

USP3 is a nuclear DUB that primarily deubiquitinates and stabilizes targets for maintenance of genome stability, regulation of cell proliferation, DNA damage response, and the innate immune response[18–23]. There are currently no selective USP3 inhibitors, due to the high conservation of the catalytic USP domain. The ZnF-UBD of USP3 is required for its interaction with and deubiquitination of targets such as histone H2A and RIG-I[18,21]. Compounds presented here are good chemical starting points for the development of protein-protein interaction inhibitors that would antagonize binding of USP3 to some of its endogenous targets. Conversely, **59** or more potent compounds may serve as chemical handles for the development of bifunctional molecules that recruit USP3 to neo-substrates and induce their deubiquitination. In addition to the identification of **59**, the focused chemical library screen also identified hits against other ZnF-UBDs, such as USP16, USP49, and BRAP (**50**, **2**, **46**, respectively) which require further validation, but may be promising starting points for hit expansion.

CONCLUSIONS

We report the identification of a USP3 ZnF-UBD ligand (**59**) that does not inhibit USP3 DUB activity. Additionally, we provide a preliminary structure activity relationship as a foundation for future improvement of the chemical series. We believe the ligands presented in this work will enable the development of future USP3-recruiting DUBTACs.

EXPERIMENTAL SECTION

Hit Expansion

Substructure search was run against the Enamine REAL database (Enamine Ltd., 2022). Compounds were clustered in ICM (Molsoft)[24] and selected to be purchased based on availability, cost, and chemical substitutions. The purity of all compounds in Table 1 was confirmed to be at least 95% as measured by LCMS.

Cloning, Protein Expression and Purification

cDNAs encoding Ub^{1-76}, Ub^{1-73}, $USP5^{171-290}$, and $HDAC6^{1109-1215}$ were cloned as previously described[12–15]. DNA encoding $USP3^{1-131}$, $USP16^{25-185}$, $USP20^{1-141}$, $USP33^{29-134}$, $USP39^{84-194}$, $USP49^{1-115}$, $USP51^{176-305}$ and $BRAP^{304-390}$ was sub-cloned into a modified vector encoding an N-terminal AviTag for biotinylation and a C-terminal His6 tag (p28BIOH-LIC), while $USP13^{183-307}$ was sub-cloned into a vector with an N-terminal His-tag and TEV protease cleavage site, and a C-terminal biotinylation sequence (pNICBIO2). $USP3^{1-520}$ was sub-cloned into a pFBOH-LIC vector and overexpressed in sf9 cells, where cultures were grown in HyQ SFX Insect Serum Free Medium (Fisher Scientific) to a density of 4 x 10^6 cells/mL and infected with 10 mL of P3 viral stock media per 1 L of cell culture. Cell culture medium was collected after 4 days of incubation in a shaker at

27ºC. Proteins were purified as before[12–15]; briefly, all proteins were purified by metal affinity chromatography, gel filtration and ion exchange chromatography. The final concentration of purified proteins was measured by UV absorbance at 280 nm. Protein identity was confirmed by mass spectrometry and purity was assessed by SDS-PAGE.

Surface Plasmon Resonance Assay

Studies were performed with a Biacore T200 (GE Health Sciences) at 20ºC. Approximately 3000-6000 response units (RU) of biotinylated ZnF-UBDs were captured to flow cells of a streptavidin-conjugated SA chip per manufacturer's protocol, and an empty flow cell used for reference subtraction. Serial dilutions were prepared in 20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.005% Tween-20 (v/v), 1% DMSO (v/v). K_D determination experiments were performed using multi-cycle kinetics with 60 second contact time, and 60 second dissociation time at a flow rate of 30 µL/min at 20 ºC. K_D values were calculated using steady state affinity fitting with the Biacore T200 evaluation software (GE Health Sciences).

Ubiquitin Rhodamine110 Assay

Experiments were performed in a total volume of 60 µL in 384-well black polypropylene microplates (Grenier). Fluorescence was measured using a Biotek Synergy H1 microplate reader (Biotek) at excitation and emission wavelengths of 485 nm and 528 nm, respectively. Ligands were prepared in 20 mM Tris pH 7.5, 125 mM NaCl, 1 mM DTT, 0.01% TX-100 (v/v), 1% DMSO (v/v) for two-fold dilution titration series. 500 nM $USP3^{1-520}$ and 200 nM ubiquitin-rhodamine110 (UBPBio) were added to each well. Following a 1-minute centrifugation at 250 g, fluorescence readings were immediately taken for ten minutes. The data was analyzed with GraphPad Prism 8.2.0.

ASSOCIATED CONTENT

Supporting Information

- Table S1: Molecular formula strings (CSV)
- Table S2: Matrix Screen results (CSV)

AUTHOR INFORMATION

Corresponding Author

Email: matthieu.schapira@utoronto.ca, rachel.harding@utoronto.ca

Author Contributions

The manuscript was written by M.K.M., and M.S. All authors have given approval to the final version of the manuscript. M.K.M expressed and purified proteins, designed and optimized biophysical assays and tested compounds., B.W conducted analytical chemistry. R.J.H. and M.S. advised throughout the project.

Funding Sources

M.S. gratefully acknowledges support from NSERC [Grant RGPIN-2019-04416], CQDM (Quantum Leap-176) and MITACS accelerate (IT13051). M.K.M acknowledges CQDM (Quantum Leap-176) and the Peterborough K.M. Hunter Charitable Foundation for financial support.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The Structural Genomics Consortium is a registered charity (no: 1097737) that receives funds from Bayer AG, Boehringer Ingelheim, Bristol Myers Squibb, Genentech, Genome Canada through Ontario Genomics Institute [OGI-196], EU/EFPIA/OICR/McGill/KTH/Diamond Innovative Medicines Initiative 2 Joint Undertaking [EUbOPEN grant 875510], Janssen, Merck KGaA (aka EMD in Canada and US), Pfizer and Takeda. We thank Dr. Vijayaratnam Santhakumar for his advice and Albina Bolotokova for compound management.

ABBREVIATIONS

BRAP, BRCA-1 associated protein; DMSO, dimethylsulfoxide; DUB, deubiquitinase; DUBTAC, deubiquitinase targeting chimera; HDAC6, histone deacetylase 6; K_D, dissociation constant; K48, lysine 48; PROTAC, proteolysis targeting chimera; SAR, structure activity relationship; SD, standard deviation; SPR, surface plasmon resonance; Ub, ubiquitin; USP, ubiquitin specific protease; ZnF-UBD, ubiquitin-specific processing protease zinc-finger

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